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Damage-Induced Phosphorylation of Sld3 is Important to Block Late Origin Firing

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Abstract

Origins of replication are activated throughout S-phase such that some origins fire early and others fire late to ensure that each chromosome is completely replicated in a timely fashion. However, in response to DNA damage or replication fork stalling, eukaryotic cells block activation of unfired origins. Human cells derived from patients with ataxia telangiectasia are deficient in this process due to the lack of a functional ataxia-telegiectasia mutated (ATM) kinase and elicit Radio-resistant DNA synthesis (RDS)^{1–3} following γ -irradiation². This effect is conserved in budding yeast, as yeast cells lacking the related kinase Mec1 (ATR) also fail to inhibit DNA synthesis in the presence of DNA damage⁴. This intra-S-phase checkpoint actively regulates DNA synthesis by inhibiting the firing of late replicating origins, and this inhibition requires both Mec1 and the downstream checkpoint kinase Rad53 (Chk2)^{5,6}. However, the Rad53 substrate(s) whose phosphorylation is required to mediate this function remained unknown. Here, we show that the replication initiation protein Sld3 is phosphorylated by Rad53, and that this phosphorylation, along with phosphorylation of the Cdc7 kinase regulatory subunit Dbf4, blocks late origin firing. Upon exposure to DNA damaging agents, cells expressing nonphosphorylatable alleles of *SLD3* and *DBF4* (*SLD3-m25* and *dbf4-m25*, respectively) proceed through S-phase faster than wild-type cells by inappropriately firing late origins of replication. *SLD3-m25 dbf4-m25* cells grow poorly in the presence of the replication inhibitor hydroxyurea (HU) and accumulate multiple Rad52 foci. Moreover, *SLD3-m25 dbf4-m25* cells are delayed in recovering from transient blocks to replication and subsequently arrest at the DNA damage checkpoint. These data suggest that the

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Author contributions L.G.D.E. identified Sld3 in initial proteomic screen. Z.O.J. and J.W. performed mass spectrometry analysis. J.L.M. performed all other experiments with assistance from N.L.M. J.L.M. and D.P.T. designed experiments and wrote the manuscript.

Methods A full description of methods is provided in Supplementary Information as Supplementary Methods. Strains and plasmids are listed in Supplementary Tables S2 and S3.

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intra-S-phase checkpoint functions to block late origin firing in adverse conditions to prevent genomic instability and maximize cell survival.

In a whole-genome screen for proteins whose electrophoretic mobility was altered by DNA damage (unpublished results), we found that Sld3, an essential protein required for the initiation of DNA replication⁷, becomes substantially modified in response to checkpoint activation. Sld3 was hyper-phosphorylated in cells treated with genotoxic drugs, but not in cells arrested at the spindle checkpoint with Nocodazole (Fig. 1a and b). The Sld3 damage-induced phosphorylation depends on Mec1 and Rad53 and is compromised in mutants that reduced Rad53 activation (Fig. 1c). DNA damage-induced Sld3 phosphorylation is not cell cycle regulated, as cells blocked in G1 or mitosis and subsequently treated with 4-nitroquinoline 1-oxide (4NQO) or Zeocin contained phosphorylated Sld3 (Fig. 1d).

Origin firing requires both the phosphorylation of Sld3 by the S-phase cyclin-dependent kinase (S-CDK)^{8,9}, and the activity of the Dbf4-dependent kinase (DDK)^{10,11}. The S-CDK phosphorylation of Sld3 allows binding to Dpb11 and promotes origin firing^{8,9}. *In vitro* binding assays using recombinant full-length Sld3 and immobilized Dpb11, revealed that S-CDK phosphorylation of Sld3 is required to bind Dpb11, as previously seen. However, subsequent Rad53 phosphorylation of S-CDK-phosphorylated Sld3 eliminated binding to Dpb11 (Fig. 1e). This indicates that Sld3 is a Rad53 substrate and suggests that, by antagonizing Sld3 binding to Dpb11, Rad53 can inhibit late origin firing.

To address this hypothesis directly, we constructed an unphosphorylatable *SLD3* allele. Using mass spectrometry, twenty-eight phosphorylation sites were identified (Supplemental Table 1a). Twenty-five potential phosphorylation sites were mutated to generate the *SLD3-m25* allele. When *SLD3-m25* was introduced into cells, damage-induced Sld3 phosphorylation was not detected (Fig. 2a and Supplemental Fig. 2). *In vitro* kinase assays revealed that activated Rad53, but not a catalytic mutant (Rad53-K227A), phosphorylated Sld3 strongly, but phosphorylated the Sld3-m25 mutant less efficiently (Fig. 2b). Additional mass spectrometry analysis of Sld3 phosphorylated *in vitro* by Rad53 identified nine phosphorylation sites, five of which were mutated in the *SLD3-m25* allele (Supplemental Table 1a).

Intra-S-phase checkpoint defective mutants, such as *rad53*, fail to prevent late origin firing in the presence of DNA damage and consequently proceed through S-phase faster than wild-type cells^{5,6}. Cells expressing *SLD3-m25* in the presence of methyl methanesulfonate (MMS) proceeded through S-phase faster than wild-type cells (Fig. 2c and d), although they progressed slower than *rad53* cells. Like Sld3, Dbf4 functions to promote firing of all origins of replication^{10,11} and is phosphorylated in response to checkpoint activation in a Rad53-dependent manner^{12,13} (Fig. 1b and 2a). Dbf4 is a periodically expressed activator of the Cdc7 kinase, known to phosphorylate the replication helicases (MCMs)^{14–17}. We reasoned that Dbf4 may also be inhibited by Rad53 to block late origin firing. Using a similar mass-spectrometry approach, six phosphorylation sites were identified on Dbf4 (Supplemental Table 1b). The six phosphorylation sites were mutated to generate a *dbf4-m6* allele, however the Dbf4 damage-induced phosphorylation shift was intact when *dbf4-m6* cells were treated with MMS (data not shown). Previous studies have shown that a Dbf4

mutant lacking the non-essential amino-terminus (1–109) is not phosphorylated in damage-treated cells¹⁸. For this reason, we mutated all serine and threonine residues in this non-essential domain of Dbf4 (except those matching the CDK consensus sequence), in addition to the six sites identified by mass spectrometry, to generate the *dbf4-m25* allele. When expressed in cells, damage-induced Dbf4-m25 phosphorylation was reduced, but not eliminated (Fig. 2a), suggesting that not all damage-induced phosphorylation sites were identified or that Dbf4 is also phosphorylated by another damage-responsive kinase(s).

Cells expressing *SLD3-m25*, *dbf4-m25*, or both mutant alleles were viable and had no growth defects at any temperature tested (Supplemental Figs. 3 and 4a). Cells expressing *dbf4-m25* alone appear to be intra-S-phase checkpoint competent (Fig. 2c and d). However, cells expressing *SLD3-m25* and *dbf4-m25* progressed faster through S-phase in the presence of MMS than cells expressing either allele alone (Fig. 2c and d and Supplemental Fig. 5), arguing that Sld3 and Dbf4 are both important for the block to late origin firing. The finding that *SLD3-m25 dbf4-m25* cells progressed through S-phase slower than *rad53* cells can best be explained by the fact the Dbf4-m25 is still partially phosphorylated *in vivo* (Fig. 2a). Damage-induced Rad53 phosphorylation and Sml1 degradation¹⁹ are intact in *SLD3-m25 dbf4-m25* cells (Fig. 2a), suggesting that these mutations do not compromise checkpoint activation.

To unambiguously determine whether the fast S-phase observed in *SLD3-m25 dbf4-m25* cells, in the presence of MMS, is due to the inappropriate firing of late origins of replication, firing was directly analyzed by two-dimensional gel electrophoresis²⁰. In HU-treated cells, early origins ARS305 and ARS607 fired efficiently and with normal kinetics in *SLD3-m25*, *dbf4-m25* and *SLD3-m25 dbf4-m25* mutants compared to wild-type cells (Fig. 3a and Supplemental Fig. 6b). This finding is consistent with early origins being refractory to intra-S-phase checkpoint regulation^{5,6}. We next looked at several late origins; ARS501, ARS609, ARS1007 and ARS1212 (Fig. 3a, Supplemental Fig. 6c and data not shown). In HU-treated cells, late origin firing, was not detected in wild-type cells, but was evident in *rad53* cells. Inappropriate late origin firing was also observed in *SLD3-m25*, *dbf4-m25* and, most strongly in the *SLD3-m25 dbf4-m25* double mutant. In these mutant cells, late origins fired after early origins, confirming the finding that the temporal regulation of origin firing is not subject to intra-S-phase checkpoint regulation (Supplemental Fig. 6b and 6c). These results suggest that the fast S-phase observed in *SLD3-m25 dbf4-m25* cells is due to the inappropriate firing of late replicating origins.

If Rad53 phosphorylation of Sld3 slows S-phase by blocking late origin firing, then constitutive Rad53 phosphorylation of Sld3 would constitutively slow S-phase, even in the absence of DNA damaging agents. To test this prediction, we created a phospho-mimetic allele, where twenty-one residues targeted by Rad53 were mutated to aspartic acids to mimic constitutive Sld3 phosphorylation (*sld3-m21D*). Cells expressing the *sld3-m21D* allele revealed a slow S-phase in the absence or presence of DNA-damaging agents (Fig. 3b and Supplemental Fig. 7a). The slow S-phase is not a result of checkpoint activation, as *sld3-m21D* cells do not elicit Rad53 phosphorylation in the absence of damage (Supplemental Fig. 7b). In addition, *sld3-m21D* cells grow with wild-type rates, are not HU-sensitive, and fire early origins of replication with efficiencies that are indistinguishable from those

observed in wild-type cells (Fig. 3c and Supplemental Fig. 7c). This suggests that Sld3 phosphorylation is sufficient to inhibit origin firing, although effects on other aspects of replication may also contribute (Supplemental Fig. 1).

An Sld3-Dpb11 fusion has previously been shown to bypass the requirement for S-CDK activity for origin firing⁹. If Rad53 phosphorylation of Sld3 functions solely to inhibit an Sld3-Dpb11 interaction, then cells expressing an Sld3-Dpb11 fusion should be intra-S-phase checkpoint defective, akin to the *SLD3-m25* allele. However, cells expressing the Sld3-Dpb11 fusion were neither intra-S-phase checkpoint defective (Supplemental Fig. 8) nor sensitive to growth on DNA damaging agents (data not shown). This suggests that, while Rad53 inhibits the Sld3-Dpb11 interaction, this inhibition is not sufficient to block late origin firing.

To test if growth of *SLD3-m25 dbf4-m25* cells is impaired by DNA damaging agents, cells were spotted on HU and MMS plates and sensitivity was compared to wild-type and *rad53* cells. *SLD3-m25 dbf4-m25* cells are mildly sensitive to the replication inhibitor HU in a dosage-dependent manner (Fig 3c and Supplemental Fig. 4b), but were not sensitive to MMS (data not shown). Interestingly, *mec1-100* cells, which are defective in the block to late origin firing, are also mildly HU-sensitive, but not MMS-sensitive^{21,22}. It is possible that *SLD3-m25 dbf4-m25* cells are sensitive to HU simply because these mutant alleles are hypomorphic. Two results suggested that this was not the case. First, the checkpoint-defective *rad53-R70,R605A* allele is epistatic to *SLD3-m25 dbf4-m25*: *SLD3-m25 dbf4-m25 rad53-R70,R605A* cells were not more HU-sensitive than *rad53-R70,R605A* cells (Supplemental Fig. 9a and 9b). Second, *SLD3-m25* is dominant to wild-type for HU-sensitivity. In HU-sensitivity experiments using heterozygous diploid cells (Fig. 3e), homozygous *SLD3/SLD3 dbf4-m25/dbf4-m25* diploid cells are resistant to HU. In contrast, heterozygous *SLD3/SLD3-m25 dbf4-m25/dbf4-m25* diploid cells are mildly HU-sensitive, similarly to homozygous *SLD3-m25/SLD3-m25 dbf4-m25/dbf4-m25* diploid cells (Fig. 3e). Similar results were obtained in flow cytometry experiments using heterozygous diploid cells (Fig. 3d). These results strongly suggest that the HU sensitivity seen in the *SLD3-m25 dbf4-m25* double mutant is not due to insufficient Sld3 activity in the *SLD3-m25* allele, but instead is due to its being refractory to Rad53 inhibition.

Previous studies have shown that wild-type cells can efficiently recover from HU arrest, whereas *rad53* cells cannot^{23,24}. We asked if *SLD3-m25 dbf4-m25* cells could recover from transient blocks to DNA replication. Wild-type, *SLD3-m25* and *dbf4-m25* single mutant cells recovered from an HU arrest and divided, entering the next cell cycle by 120 minutes (Fig. 4a). However, only a fraction of *SLD3-m25 dbf4-m25* cells appeared to recover immediately from an HU arrest by 120 minutes; the majority remained arrested in mitosis, suggesting that these cells harbored DNA damage (Fig. 4a). We then examined Rad53 de-phosphorylation kinetics. In wild-type, *SLD3-m25*, and *dbf4-m25* cells, Rad53 became phosphorylated during the HU arrest and was de-phosphorylated after the HU release. In contrast, *SLD3-m25 dbf4-m25* cells retained Rad53 phosphorylation for 120 minutes after HU release (Fig. 4b). While it is likely that inappropriate firing of late origins leads to a proportional increase in fork collapse, Sld3 phosphorylation likely provides additional HU resistance through other uncharacterized means.

Rad52 functions in recombinational repair and can potentially repair or restart broken or stalled replication forks. Rad52-GFP foci have been shown to form in response to replication fork collapse during S-phase in several checkpoint mutants^{25,26}. In wild-type cells, however, foci were not observed when cells are treated with either HU or MMS^{25,27}. Consistently, we observed Rad52-GFP foci in 3% of wild-type and 53% of *rad53* cells treated with HU. Interestingly, 32% of *SLD3-m25 dbf4-m25* cells treated with HU formed Rad52-GFP foci (Fig. 4c) with some cells containing multiple foci (Supplemental Figs 10, 11, and 12). We also observed that *SLD3-m25 dbf4-m25* double mutants cells exhibited a 50% viability loss after a 4 hour HU treatment (Fig. 4d). Consistent with the observed increase in Rad52 foci, the mild viability loss seen in *SLD3-m25 dbf4-m25* cells after transient HU treatment is exacerbated when *RAD52* is also deleted. This suggests that in HU, *SLD3-m25 dbf4-m25* cells repair by homologous recombination to survive. As expected, *rad53* cells show a severe viability loss after HU treatment due the additional roles of Rad53 in fork stabilization^{23,24}. Taken together, these data suggest that failure to block late origins in the presence of replication inhibitors results in an increased number of stalled replication forks, and perhaps increases the propensity of replication fork collapse. Rad52-dependent pathways may function to resolve or restart these stalled forks and its loss likely generates problems at the fork that ultimately contribute to cell lethality.

DNA replication and the intra-S-phase checkpoint machinery are conserved in organisms ranging from yeast to humans. Recently several vertebrate candidate Sld3 orthologues have been identified, including Treslin/Ticcr, GEMC1 and DUE-B²⁸. Of these candidates, Treslin/Ticcr shares significant domain conservation with Sld3²⁹. It remains to be seen if ATM/ATR or Chk2 also inhibit any of these new DNA replication factors upon DNA damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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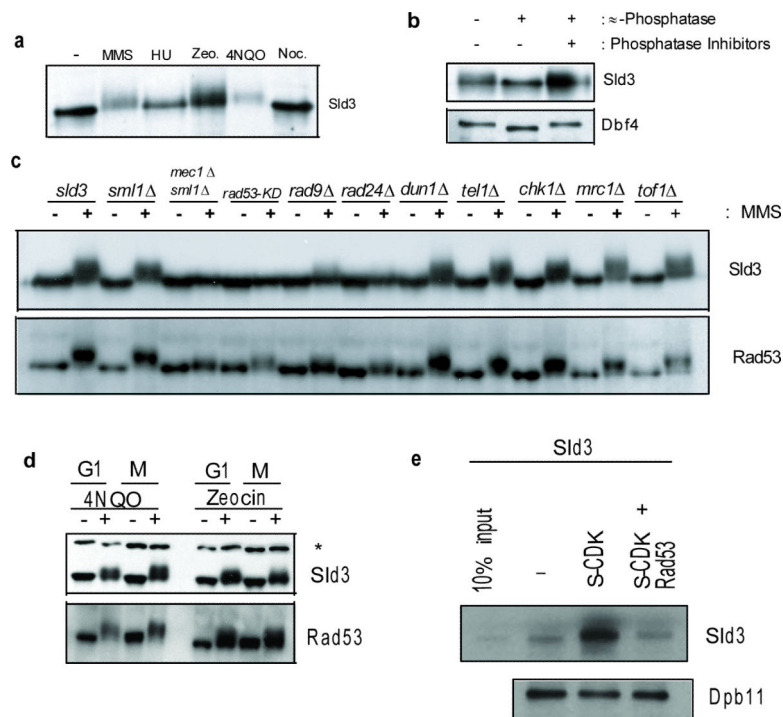


Figure 1. Rad53-dependent phosphorylation of Sld3

a, Immunoblotting of asynchronous cells expressing Sld3-3Flag in the absence (–) or presence of MMS (alkylating agent-0.05%), HU (ribonucleoside reductase inhibitor-200 mM), zeocin (radiation mimetic-200 µg/mL), 4NQO (UV-mimetic-2 µg/mL), or nocodazole (10 µg/mL) for 90 minutes. **b**, Phosphatase assays of Sld3-3Flag and Dbf4-TAP purified from MMS-treated cells. **c**, Immunoblot of checkpoint deletion mutants expressing Sld3-9myc. Samples were taken from cells arrested in G1 with α -factor and released in the presence or absence of MMS for 180 minutes. **d**, Immunoblot of cells expressing Sld3-9myc arrested in G1 with α -factor or metaphase with nocodazole for 120 minutes and then treated with either 4NQO or Zeocin, as in **a**, for 60 minutes. * Denotes a background band. **e**, *In vitro* binding assay. Recombinant Sld3-3Flag mock phosphorylated (–), Clb5-TAP CDK phosphorylated or Clb5-TAP CDK phosphorylated followed by Rad53 phosphorylation. 10% of the untreated Sld3-3Flag is shown as input. Immobilized Dpb11-TAP was used to pull down the differentially phosphorylated Sld3 species.

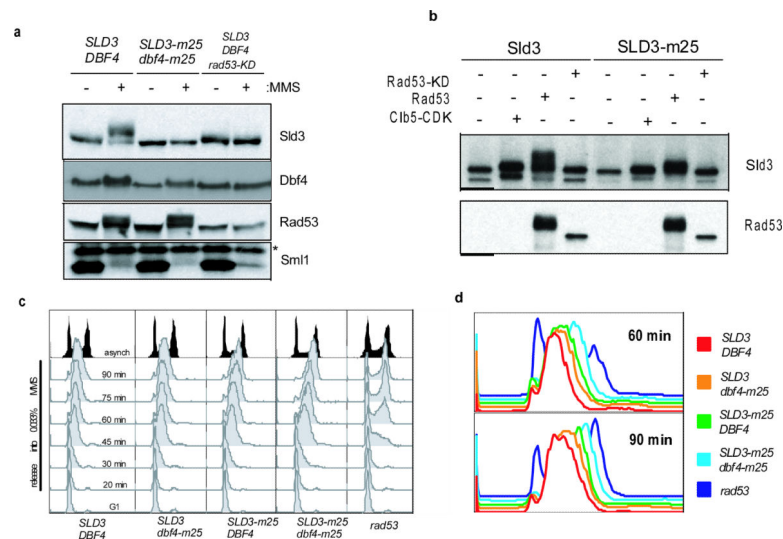


Figure 2. *SLD3-m25* and *dbf4-m25* cells are intra-S-phase checkpoint defective

a, Immunoblots from asynchronous cells grown in the presence or absence of 0.05% MMS for 90 minutes. *rad53-KD* (*K227A*) is a hypomorphic allele that is checkpoint-defective, but able to perform the essential function rescued by *Smi1* deletion. **b**, Immunoblots of an *in vitro* kinase assay on recombinant full-length Sld3 or Sld3-m25 substrates treated with yeast clb5-TAPCDK, recombinant Rad53 or Rad53-KD (*K227A*). **c**, Flow cytometry of wild-type, *SLD3-m25*, *dbf4-m25*, *SLD3-m25 dbf4-m25*, or *rad53* cells synchronized in G1 with α -factor and released into 0.033% MMS at 30°C. **d**, The 60 and 90 minute profiles from **c**, displayed together.

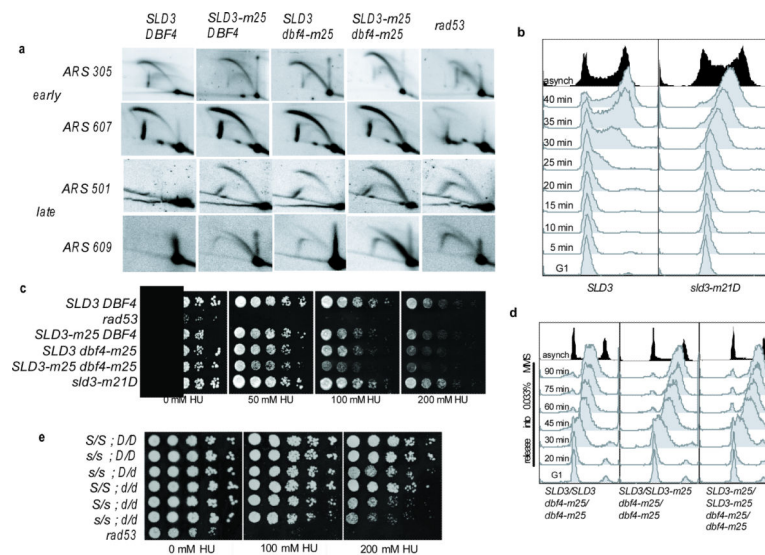


Figure 3. Inappropriate firing of late origins in the presence of replication inhibitors

a, Two-dimensional gel analysis of replication intermediates. Wild-type, *SLD3-m25*, *dbf4-m25*, *SLD3-m25 dbf4-m25* or *rad53* cells were synchronized with α -factor and released into 200 mM HU. Cells were collected at 30, 40, 50, 60 and 70 minutes into the HU release and pooled. 20 μ g aliquots of DNA were analyzed by two-dimensional electrophoresis as previously described²⁰. early origins; *ARS305* and *ARS607*. late origins; *ARS501* and *ARS609*. **b**, Flow cytometry of cells expressing *SLD3-3Flag* or a phosphorylation-mimetic allele *sld3-m21D-3Flag*. Cells were arrested in G1 with α -factor and released into media without α -factor at 30°C. **c**, 5-fold serial dilutions of wild-type or mutant strains grown on 0 mM, 50 mM, 100 mM, or 200 mM HU. **d**, Flow cytometry of diploid MAT a/a cells treated as in **2c**, from *SLD3/SLD3 dbf4-m25/dbf4-m25*, *SLD3/SLD3-m25 dbf4-m25/dbf4-m25*, and *SLD3-m25/SLD3-m25 dbf4-m25/dbf4-m25* cells. **e**, 5-fold serial dilutions, as in **c**, of diploid strains to assess dominance. Capital **S** and **D** represent wild-type *SLD3* and *DBF4*, whereas lowercase **s** and **d** represent *SLD3-m25* and *dbf4-m25* respectively.

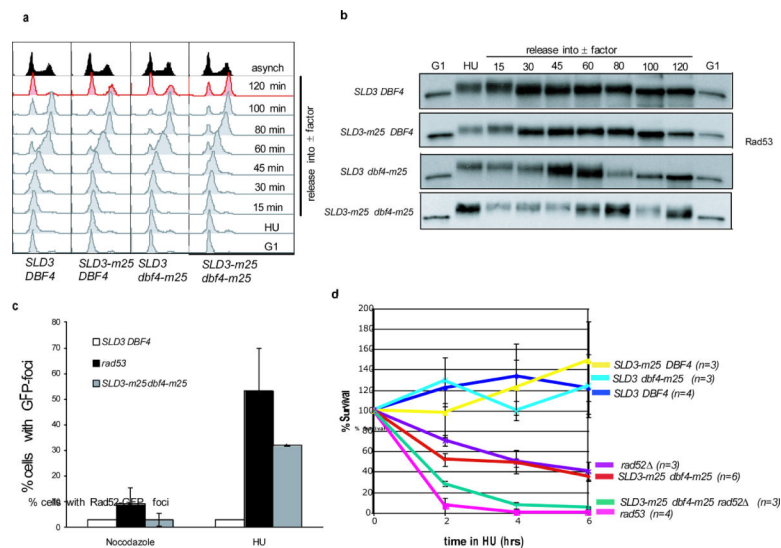


Figure 4. Inappropriate firing of late origins elicits DNA damage

a, Flow cytometry of wild-type or mutant strains synchronized in G1 with α -factor, released into a 200 mM HU block for 2 hours, then released into medium with α -factor. **b**, Immunoblots of samples from **a**, taken at indicated time points and probed for Rad53. **c**, Quantification of cells with one or more Rad52-GFP foci after incubating for 90 minutes in the presence of nocodazole or 200 mM HU. Error bars represent s.e.m.; $n = 3$. **d**, Survival assay of strains synchronized in G1 and released into 200 mM HU for 2, 4 or 6 hours. Error bars represent s.e.m.; n is indicated on graph.